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**Full title**

Th17 responses to pneumococcus in blood and adenoidal cells in children

**Short Title**

Th17 responses to pneumococcus in children

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## 1. **Summary**

Pneumococcal infections cause a large global health burden and the search for serotype-independent vaccines continues. Existing conjugate vaccines reduce nasopharyngeal colonisation by target serotypes. Such mucosal effects of novel antigens may likewise be important. CD4<sup>+</sup> Th17 cell-dependent, antibody-independent reductions in colonisation and enhanced clearance have been described in mice. Here we describe the evaluation of Th17 cytokine responses to candidate pneumococcal protein vaccine antigens in human cell culture, using adenoidal and peripheral blood mononuclear cells. Optimal detection of IL-17A was at day 7, and of IL-22 at day 11, in these primary cell cultures. Removal of CD45RO<sup>+</sup> memory T cells abolished these responses. Age-associated increases in magnitude of responses were evident for IL-17A, but not IL-22 in adenoidal cells. There was a strong correlation between individual IL-17A and IL-22 responses after pneumococcal antigen stimulation ( $p < 0.015$ ). Intra-cellular cytokine staining following PMA/Ionomycin stimulation demonstrated that >30% CD4<sup>+</sup> T cells positive for IL-22 express the innate markers  $\gamma\delta$ T-cell receptor and/or CD56, with much lower proportions for IL-17A<sup>+</sup> cells ( $p < 0.001$ ). Responses to several vaccine candidate antigens were observed but were consistently absent, particularly in blood, to PhtD ( $p < 0.0001$ ), an antigen recently shown not to impact colonisation in a clinical trial of a PhtD-containing conjugate vaccine in infants. The data presented, and approach discussed have the potential to assist in the identification of novel vaccine antigens aimed at reducing pneumococcal carriage and transmission thus improving the design of empirical clinical trials.

## 2. Introduction

*Streptococcus pneumoniae* (pneumococcus) remains a significant global cause of morbidity and mortality from diseases including pneumonia, meningitis, sepsis and otitis media, and is a particular challenge in developing countries. Pneumococcus accounts for 11% of all deaths in children under 5 years of age resulting in up to a million childhood deaths every year (1, 2). Of growing global concern are the emergence of non-vaccine serotypes, and antibiotic-resistant strains of pneumococcus (3, 4).

Pneumococcus is a commensal of the human upper respiratory tract, with more than 90 capsular serotypes described. Asymptomatic colonisation of the nasopharynx with pneumococcus is more common in young children than in older children and adults (5-7). In contrast, the development of invasive pneumococcal disease is, relatively speaking, rare. Transmission between children and to other family members sustains the bacteria within a community (8). The introduction of multivalent pneumococcal conjugate vaccines (PCV) has resulted in overall decreases in the incidence of pneumococcal disease, but overall colonisation rates in children have changed little as non-vaccine serotypes replace formerly dominant vaccine types and also cause some replacement disease, limiting the overall efficacy of the vaccines in some settings (9-11). Thus, the development of serotype-independent pneumococcal vaccines is a priority in the fight against pneumococcus.

If, like PCVs, such vaccines are to impact disease by reducing carriage and transmission, understanding of naturally-acquired mucosal immune responses to pneumococcus and how they affect pneumococcal colonisation could guide antigen selection and vaccine formulation. Antibody-independent CD4<sup>+</sup> T cell-dependent reduction of pneumococcal colonisation has been demonstrated in mice (12-14). A role for CD4<sup>+</sup> Th17 cells, which can kill and clear pneumococci by recruiting neutrophils to the site of infection, has been proposed (15, 16). Th17 cells exist in adults and children (15), and produce both interleukin-17A (IL-17A) and interleukin-22 (IL-22) (17, 18). The stimulation of production of IL-17A and IL-22 by candidate pneumococcal vaccine antigens could indicate their capacity to influence pneumococcal colonisation either by preventing acquisition or promoting clearance.

Here we describe the measurement and characteristics of Th17 responses in human primary cell cultures from blood and adenoidal tissue (nasal associated lymphoid tissue) of children, and the use of this technique to screen potential pneumococcal vaccine candidate antigens. In particular, we show that for an antigen that recently failed to reduce carriage in a clinical trial, no demonstrable Th17 responses could be seen.

### **3. Materials and methods**

#### **3.1 Subjects and samples**

With informed consent, adenoids were collected from children aged 1-14 years, who were undergoing routine adenoidectomy or adenotonsillectomy at the Bristol Royal Hospital for Children. Up to 10mL of anti-coagulated peripheral blood was additionally collected from some children. Children were healthy at the time of surgery. Exclusion criteria were: recent/current antibiotics, known immunodeficiency or immunosuppressive treatment within 2 weeks of surgery. Clinical information is provided in detail in Supplementary table 1. They had previously received 7 valent pneumococcal conjugate vaccine almost without exception, and it should be noted that the majority of children were aged 3 years and above. The total number of children studied was 75, and the numbers in the comparisons described varied between 3 and 38. Amongst those from whom a nasal swab was obtained and analysis completed (n=61), the pneumococcal carriage rate by culture was 39%. Ethical approvals were obtained as appropriate from the North Somerset and South Bristol and PATH Research Ethics Committees.

### 3.2 Antigens

The whole-cell killed unencapsulated pneumococcal antigen (WCA) was made as described in (19) at a concentration of killed bacteria representing  $1 \times 10^6$  cfu/ml, as determined in previous experiments (data not shown). Different WCA preparations have been shown to be similar with respect to specific antigen content, and induction of immunological responses in laboratory animals. Recombinant proteins Choline binding protein A (CbpA), Pneumococcal surface antigen A (PsaA) and Pneumococcal surface protein A (PspA) were purified from

recombinant *E.coli* expressing the respective cloned genes (20, 21). Recombinant CbpA, PsaA and PspA were assessed to be >95% pure by SDS-PAGE and Coomassie brilliant blue staining. Recombinant protein Pneumococcal histidine triad protein D (PhtD) was produced as previously described (22), and isothermal calorimetry was performed to confirm zinc binding with ID NMR to show associated changes in protein structure. All proteins were used at a concentration of 8µg/ml to stimulate optimal CD4<sup>+</sup> T cell proliferation as determined in previous experiments, as shown in supplementary figure 1. The duration of cell stimulation varied between experiments and is shown in the respective figure legends.

### 3.3 Cell isolation and culture

Adenoidal tissue was collected into Hanks' Balanced Salt Solution (HBSS)/ 2% Hepes (Thermo Scientific/Life Technologies, USA and Sigma-Aldrich Company Ltd., UK) with 10% Penicillin/Streptomycin (Sigma-Aldrich Company Ltd., UK), and processed fresh within 24 hours. Peripheral blood was processed within 6 hours of collection and was prepared by diluting it 1:1 with HBSS/2% Hepes. Mononuclear cells from adenoids and peripheral blood were separated on Ficoll-density gradients as previously described (23). Cells were cultured in 48-well culture plates at 1x10<sup>6</sup> cells/ml in a 1 mL volume, in either complete Roswell Park Memorial Institute Medium 1640 (RPMI) (Thermo Scientific/Life Technologies, USA) containing 20mM Hepes, 2mM L-Glutamine, 1% Penicillin/Streptomycin (Sigma-Aldrich Company Ltd., UK) with 10% Foetal bovine serum (Sigma-Aldrich



Company Ltd., UK) for cytokine analysis, or in complete RPMI media/2% human serum (Sigma-Aldrich Company Ltd., UK) for intra-cellular cytokine analysis.

### 3.4 Cell depletions

Memory T cells (CD45RO+) were depleted from the mononuclear cell population using positive selection magnetic-activated cell sorting (MACS) according to the manufacturer's guidelines (Miltenyi Biotech, Germany). A positive control ("add back") was made by mixing the depleted cells with the positively selected cells retained on the magnet during the cell separation procedure. The purity of these cell suspensions (CD45RO- and CD45RO+) was confirmed by immunofluorescence staining (CD4-APC, CD45RO-FITC and CD45RA-PE-Cy7 (BD Biosciences, UK)) and flow cytometry using a FACS Canto II (BD Biosciences, UK), analysed with FlowJo software (FlowJo, LLC, USA) analysis was conducted by gating on lymphocyte cells identified by their forward/side scatter profile, gating the CD4+ population and finally gating on CD45RO and CD45RA cell populations. CD45RO and CD45RA cells were re-mixed at a 1:1 ratio (proportion of CD45RO cells before depletion was 16-38%, and <1% after depletion).

### 3.5 IL-17A and IL-22 immunoassays

Cells were incubated with or without antigen, and IL-17A and IL-22 was measured in the cell supernatant collected between 1-15 days using a Human IL-17A or an IL-22 ELISA Ready-Set-Go kit according to the manufacturer's instructions

(Affymetrix eBiosciences,USA). In most cases the same cell supernatant was used for both cytokine assays, except for the experiments shown in figures one and two. Supernatants were stored at -20°C for short term and at -80°C for long term storage.

### 3.6 Intra-cellular cytokine production

Intra-cellular cytokine staining was conducted on day 7 of cell culture with or without antigen stimulation. Cells were re-stimulated with antigen on day 6 to boost their cell specific cytokine responses. On day 7 PMA 0.05 µg/ml, Ionomycin 1 µg/ml (Sigma-Aldrich Company Ltd., UK) and Golgistop (BD Biosciences, UK) were added for 5 hours. Cell viability staining, cell surface staining and the intracellular cytokine staining processes were carried out using a BD Cytofix/Cytoperm Fixation/Permeabilization Kit according to the manufacturer's instructions (BD Biosciences, UK). Fixable viability dye eFluor780 (Affymetrix eBiosciences,USA) was used to assess cell viability. Cell surface antibody markers CD4 Alexa Fluor700 (BD Biosciences, UK), CD56 PE-Cy7/ Brilliant Violet 510 (BD Biosciences, UK/ Biolegend, USA), TCR γδ FITC/ PE-Cy5.5 (BD Biosciences, UK/ Beckman Coulter, USA) and intracellular antibodies IL-17A PE/ Brilliant Violet 605 (Affymetrix eBiosciences,USA/ Biolegend, USA) and IL-22 eFluor660 (Affymetrix eBiosciences,USA) were used and concentrations had been pre-determined by titrations. The fixed and stained cells were left overnight at 4°C to reduce autofluorescence before being analysed on a LSR II flow cytometer (BD Biosciences, UK), where 20,000 live cells in the lymphocyte gate

were collected per stimulation. Analysis was carried out using the software program FlowJo, and only live cells in the lymphocyte gate were analysed. Analysis was conducted by gating on lymphocyte cells identified by their forward/side scatter profile, live cells were gated based on the live/dead cell marker, then either IL-17A+ or IL-22+ populations were identified, followed by the CD4+ population and finally the CD56+ or  $\gamma\delta$ TCR+ cell populations were gated on.

### 3.7 Statistical analysis

Significance of differences between groups was analysed using a paired t-test. The relationships between age and cytokine responses was compared using linear regression analysis. Pearson correlation was used to investigate correlation in an individual's IL-17A and IL-22 cytokine response to an antigen. Group mean cytokine responses to the panel of antigens were compared by repeated measures one-way ANOVA. Statistical analysis was performed using GraphPad Prism version 6 (GraphPad Software, USA).

## **4. Results**

### **Optimisation of assay system and identification of responding cells**

In order to determine optimal methodology for detecting responses, time course experiments were initially conducted using peripheral blood mononuclear cells

(PBMC), and adenoidal mononuclear cells (AMNC) stimulated with WCA. The Th17 cytokines IL-17A (Figure 1a) and IL-22 (Figure 1b) responses, when present, were consistently strong at days 7 and 11 respectively and these timings were used in subsequent experiments.

To identify the phenotype of responding cells, two experiments showed that depletion of memory T cells (CD45RO+) from PBMC almost entirely eliminated detectable IL-17A responses (Figure 2.a), which were restored by their replacement (Figure 2.b). This finding was confirmed in four additional children whose undepleted PBMC IL-17A responses were median (range) 40.5 (6.3-65.6) pg/ml on day 5, and CD45RO-depleted (99% purity in all) IL-17A responses 0 (0-0.4) pg/ml (data not shown).

### **Characterisation of cytokine responses**

Experiments were performed to establish the key characteristics of these responses. There was a correlation between age and the IL-17A responses to WCA in AMNC, but little evidence that age influenced the IL-22 response in AMNC or the IL-17A or IL-22 response in PBMC. (Figure 3). There was a strong positive correlation in the magnitude of IL-17A and IL-22 responses to WCA and CbpA in individual subjects in both PBMC and AMNC (Figure 4).

Intracellular cytokine staining was used to investigate further the phenotype of cells producing IL-17A and IL-22. For intracellular cytokine staining, IL-22 detection was measured after 7 days rather than the optimal day 11 (Figure 5), as there were limited available cells. Live lymphocyte gating (based on forward

and side scatter parameters) in flow cytometric staining for intracellular IL-17A and IL-22 showed strong evidence of increases of 2% or more above the background following stimulation with the recombinant pneumococcal antigen CbpA ( $p=0.0021$ , *paired t test*) (Figure 5.a).

Fifty percent or more IL-17A+ live lymphocytes were CD4 positive, with a somewhat lower percentage of IL-22+ AMNC expressing CD4 (Figure 5. b). Following stimulation with WCA there was strong evidence of an expansion in the CD4+ IL-17A+ AMNC expressing compared to cultured cells that were stimulated with only PMA/Ionomycin.

There was very strong evidence that a much higher proportion of IL-22+ CD4+ AMNC expressed one, the other or both of the innate cellular markers CD56 and TCR- $\gamma\delta$ , than IL-17A+ CD4+ AMNC both before and after antigen stimulation (Figure 6).

### **IL-17A and IL-22 responses to a panel of pneumococcal antigens**

In order to compare responses to different candidate vaccine antigens, cytokine release by PBMC and AMNC following stimulation with pneumococcal antigens, including 3 additional proteins previously investigated as candidate vaccines, were measured. Results showed significant variation between antigens (Figure 7), and in particular there was little apparent response to the surface protein PhtD in PBMC, while responses to the additional proteins PsaA and PspA were weak in AMNC.

Additional post-hoc analysis, added during the peer review process (supplementary figures 3 and 4), indicated apparent significant differences between WCA and the recombinant proteins tested, apart from between WCA and CbpA in adenoids and differences between PhtD and PspA and PsaA in blood but not clearly in adenoids.

## **5. Discussion**

We have defined IL-17A and IL-22 responses to pneumococcal antigens in adenoidal and peripheral blood mononuclear cells, and thus propose a tool with which to evaluate Th17 cellular immune responses to candidate pneumococcal vaccine antigens. This may assist prioritization for assessment of their effects on colonisation and their potential prevention of transmission.

Previous Th17 experiments in mice found day 3 to be optimal to detect IL-17A responses, and IL-17A can also be detected from human pharyngeal tonsil mononuclear cells after 3 days (15). However, we have previously shown that CD4<sup>+</sup> T cell proliferation in response to pneumococcal antigens in children is optimal after 7 days of stimulation in culture (24) and likewise in the experiments presented here, 7 days of culture was optimal for IL-17A detection while 11 days was the best for IL-22 detection (Figure 1). We have previously demonstrated clearer and more reliable mucosal responses in adenoidal than tonsillar cells (25). Although these relatively slow response rises might suggest that these are not memory responses following previous exposure, depletion of CD45RO<sup>+</sup> cells

resulted in their almost complete disappearance (Figure 2), confirming that they are anamnestic, albeit not extremely rapid, in concordance with previous studies of other aspects of these mucosal cellular immune responses (24).

Both rates of colonisation and invasive disease due to pneumococcus drops rapidly with increasing age in young children (5-7), suggesting progressive rises in specific immunity either in response to exposure, or through immune maturation or both. Both we (5) and others have shown evidence of emergence of specific B cell immunity to pneumococcal antigens particularly during the second year of life (7, 26). IL-17A responses to pneumococcal antigens in blood leukocytes are higher in adults than children and vary between children in different populations, which is likely to reflect different levels of exposure to pneumococcus (15, 27). In the results presented here, there was little evidence of increasing Th17 responses with age, apart from rises in adenoidal cellular elaboration of IL-17A in response to pneumococcal stimulation (Figure 3). The IL-22 responses of individual children in blood and adenoids are not significantly correlated to each other, but IL-17A responses are (supplementary figure 2), and close inspection of the data (figure 3b) suggests that the reason the IL-17A age effect is not seen in blood lymphocytes is the more consistently detectable responses in the younger children studied. It is also possible that clearer age-dependency was not seen in this study in the context of wide variation between individuals owing to other potentially confounding factors including timing of recent exposure and colonisation. As noted, previous studies by our group in this paediatric population showed a consistent steep rise in antibodies to a

range of pneumococcal antigens during the second year of life (5), suggesting that few if any of the children reported here are likely never to have been colonised or that serology done in this group would be likely to predict or explain the differences we observed. Alternatively, or in addition, much of any cytokine increase may occur by the end of the second year of life as seen for antibodies to pneumococcal antigens (5), while the children studied here were nearly all aged 2 years or older.

The classic Th17 pathway shows production of IL-17A and IL-22 to be from CD4+ T cells under the influence of TGF- $\beta$ , IL-6 and IL-23 (15, 28-30). Mucosal immunity to colonisation by pneumococcus can be antibody independent and CD4+ T cell-dependent in mice (14, 15), and CD4+ T cells may be important in protection of humans against pneumococcal colonisation and disease. For example in HIV-infected individuals with reduced numbers of CD4+ T cells and high risk of this infection (31). The question then arises whether CD4+ T cells are an important cellular source of IL-17A and IL-22. While it was clear that both cytokines were elaborated by cells falling within the lymphocyte scatter gate, our results suggest that CD4+ T cells are a source of IL-17A following stimulation with pneumococcal antigens, but this was not clearly demonstrated for IL-22 under the conditions we used (Figure 5). Nevertheless, we did show evidence of strong correlation at an individual level between the size of IL-17A and IL-22 responses measured after pneumococcal antigen stimulation both in AMNC and PBMC (Figure 4). Further characterisation of CD4+ T cells expressing the two cytokines both before and after pneumococcal antigen stimulation showed that those



producing IL-22 were much more likely to be expressing innate cell phenotypes (Figure 6). Both innate NK T cells (32, 33) and TCR-  $\gamma\delta$  cells (34, 35) have previously been shown to be sources of IL-17A and IL-22, and both these cell types have been shown to recruit neutrophils to the pneumococcal infection site (36, 37). Our results extend these observations to include CD4<sup>+</sup> T cells expressing innate markers as a potential source of IL-22, particularly in the human upper respiratory tract. In this context it would be of interest to conduct further CD45RO depletion experiments to examine the effect on IL-22 production.

We and others have previously investigated mucosal and systemic B cell and T cell CD4<sup>+</sup> responses to several pneumococcal antigens including WCA, CbpA, PsaA and PspA (5, 12, 23, 24, 38). These antigens have also been shown to have protective effects in murine models of pneumococcal colonisation and infection (39-41). There has been strong interest in PhtD as a vaccine candidate antigen (22). The IL-17A and IL-22 responses we demonstrate here to these antigens in primary human cell cultures, notwithstanding wide inter-individual variation, demonstrate significance differences between antigens as well (Figure 7). The relatively larger responses seen in blood than adenoidal cells occur in the context of much lower background cytokine production by unstimulated cultures and a known lower T-regulatory environment and has previously been observed by us and others (42) (43), however further investigation is required to further understand this observed difference. Of particular note were the relative lack of responses to PhtD evident in blood, an antigen which has recently been shown to lack efficacy against pneumococcal colonisation in children although when

injected parenterally with aluminium rather than a T-cell adjuvant (44). Although it has been proposed that this and related pneumococcal proteins, which are released extra-cellularly by the bacterium in large quantities, might act as a sink for potentially opsonophagocytosing antibodies (45), our data suggest PhtD may also fail to induce cellular immune responses.

We here describe Th17 responses to pneumococcal antigens in human cell cultures in detail. This approach not only allows detailed description of the immunological responses to pneumococcus in the upper respiratory tract of children, but also has potential to guide antigen selection for candidate vaccines aiming to impact upon carriage and transmission. Future studies should seek to elucidate whether such responses reliably predict protection against acquisition or clearance of carriage in children having repeated evaluation of colonisation over time.

## **6. Acknowledgments**

EO and CP conducted the experiments. AF, EC, CP and EO designed the experiments. CLH assisted providing samples. AO, JCP, TM and RM provided the antigens. EO and AF wrote the paper with assistance of all the other authors.

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and our nurses Phoebe Mouldsdales, Clare Harrison and Jo Jenkins for recruiting the children to the study. We also acknowledge the assistance of Dr. Andrew Herman and the University of Bristol Faculty of Biomedical Sciences Flow Cytometry Facility.

## **7. Conflict of interest**

RM is a named inventor on a patent describing the use of the pneumococcal whole cell vaccine, and is the scientific founder as well as member of the board of directors of Affinivax, a company that is developing a pneumococcal vaccine.

JCP is a co-founder and director of GPN Vaccines Pty Ltd, a company that is developing a pneumococcal vaccine.

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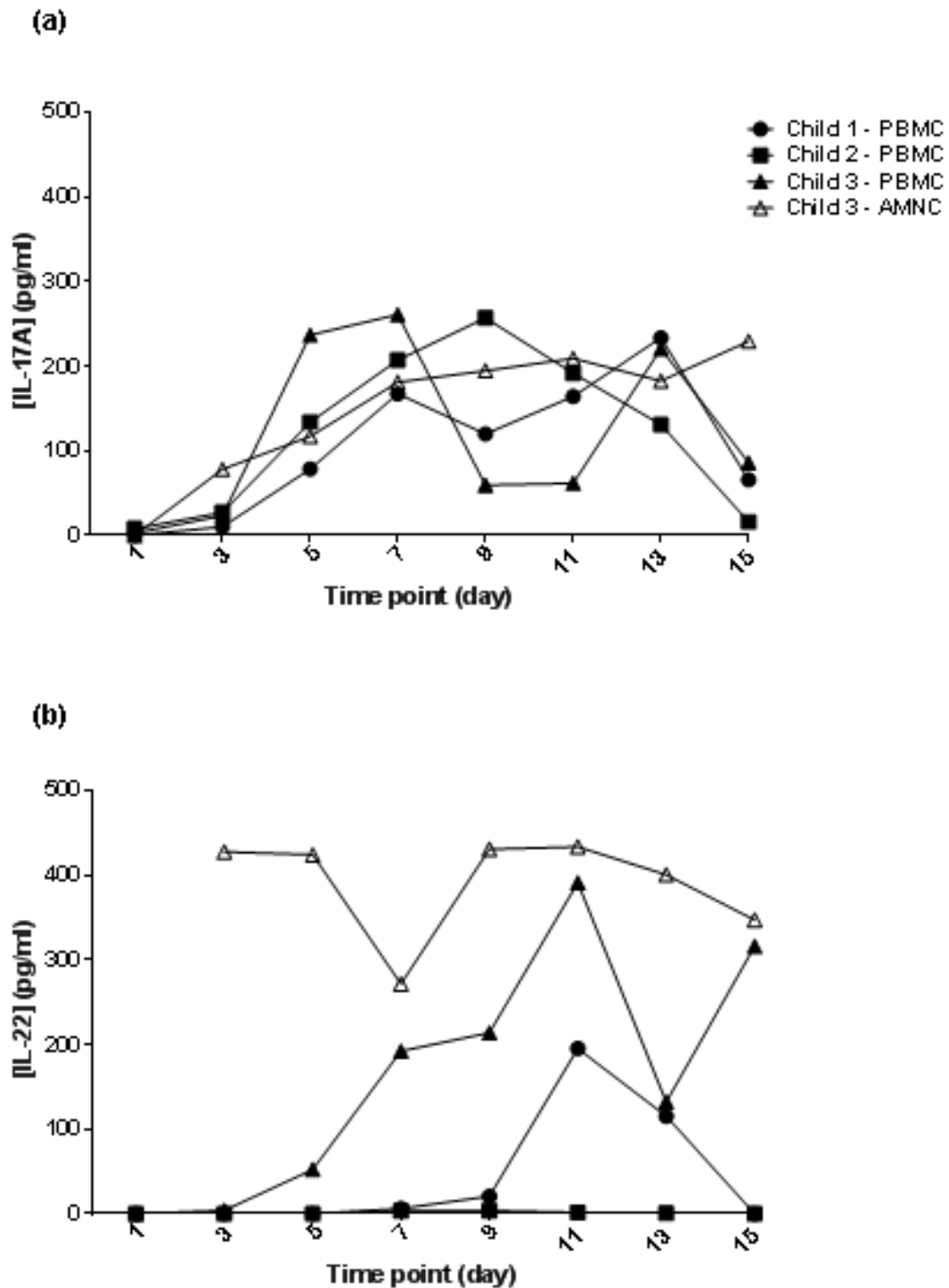
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## Figures and figure legends

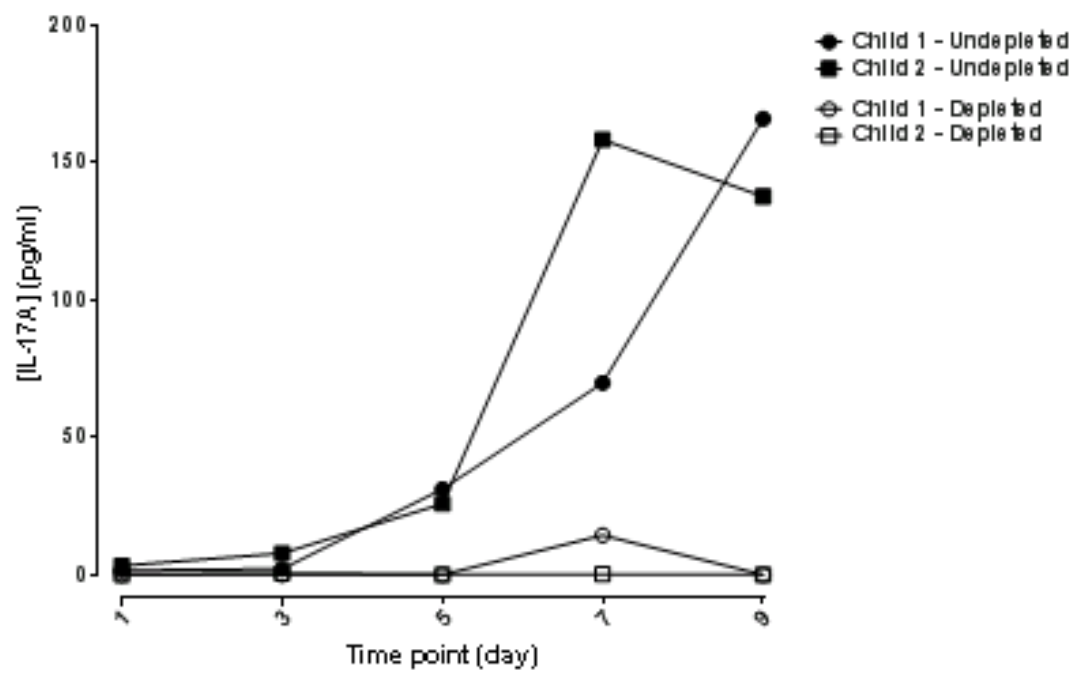


**Figure 1.** Detection of Th17 cytokines (a) IL-17A and (b) IL-22 in three children's PBMC, and in one child's AMNC after WCA stimulation over a 15 day time course. Each data point represents a child's cytokine response with the background

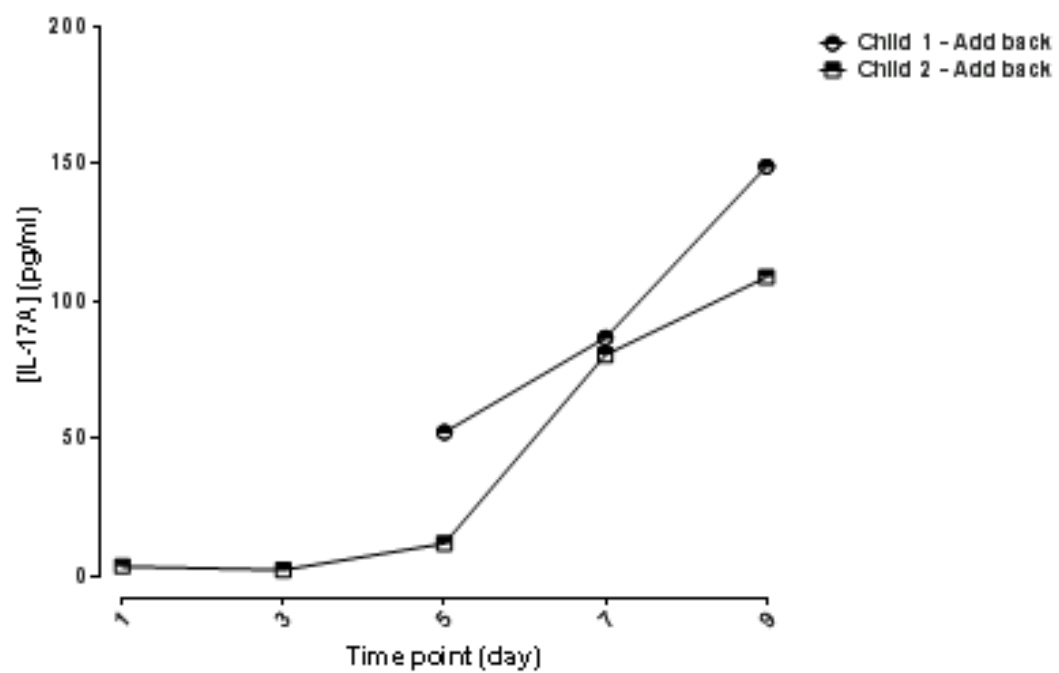
signal subtracted. Each supernatant was taken from a different well of cells at each time point. The PBMC IL-17A response for child 3 was anomalous at days 9 and 11, however the response at day 7 fitted the broader trend.



(a)

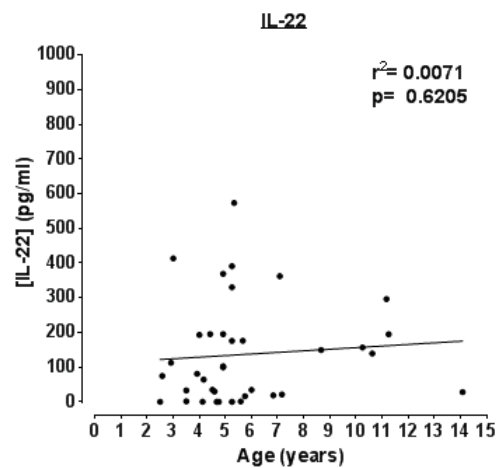
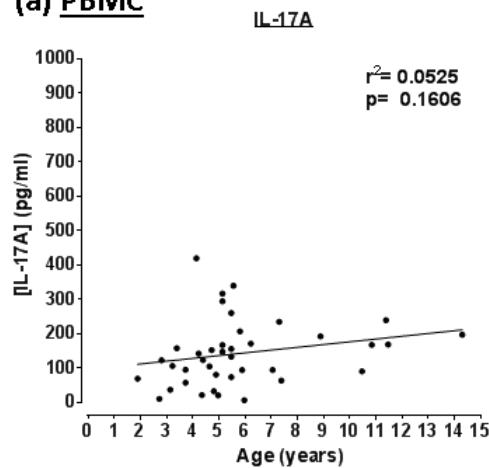


(b)

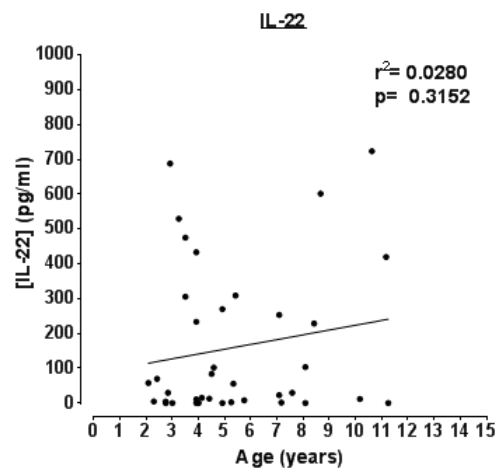
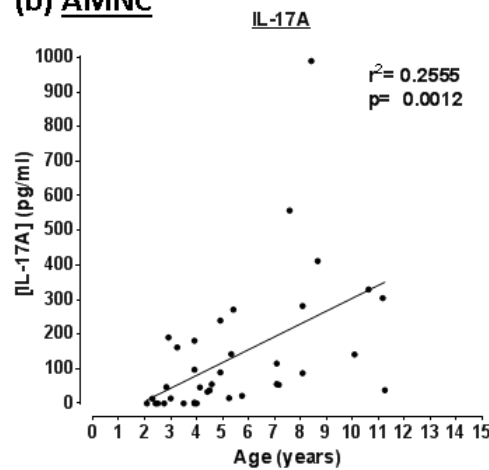


**Figure 2.** IL-17A responses to WCA over 9 days (a) in undepleted PBMC and memory T cell-depleted (CD45RO-) PBMC, and (b) in memory T cell-depleted PBMC with the memory T cells added back. CD45RO-depleted cells were 99.93% and 99.78% pure (data not shown). For child 1 the add back experiment was only conducted between days 5 and 9 due to limited cells numbers. At each time point supernatants from different wells were used and values are shown with unstimulated cells signals subtracted.

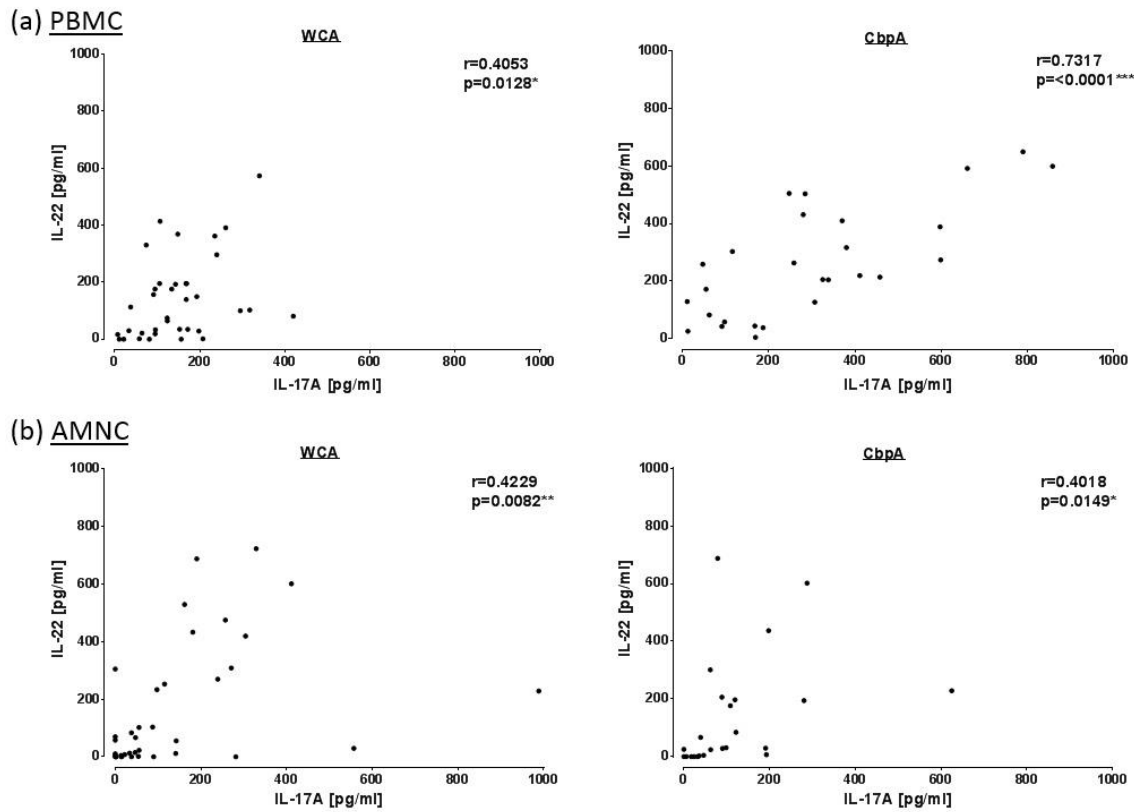
**(a) PBMC**



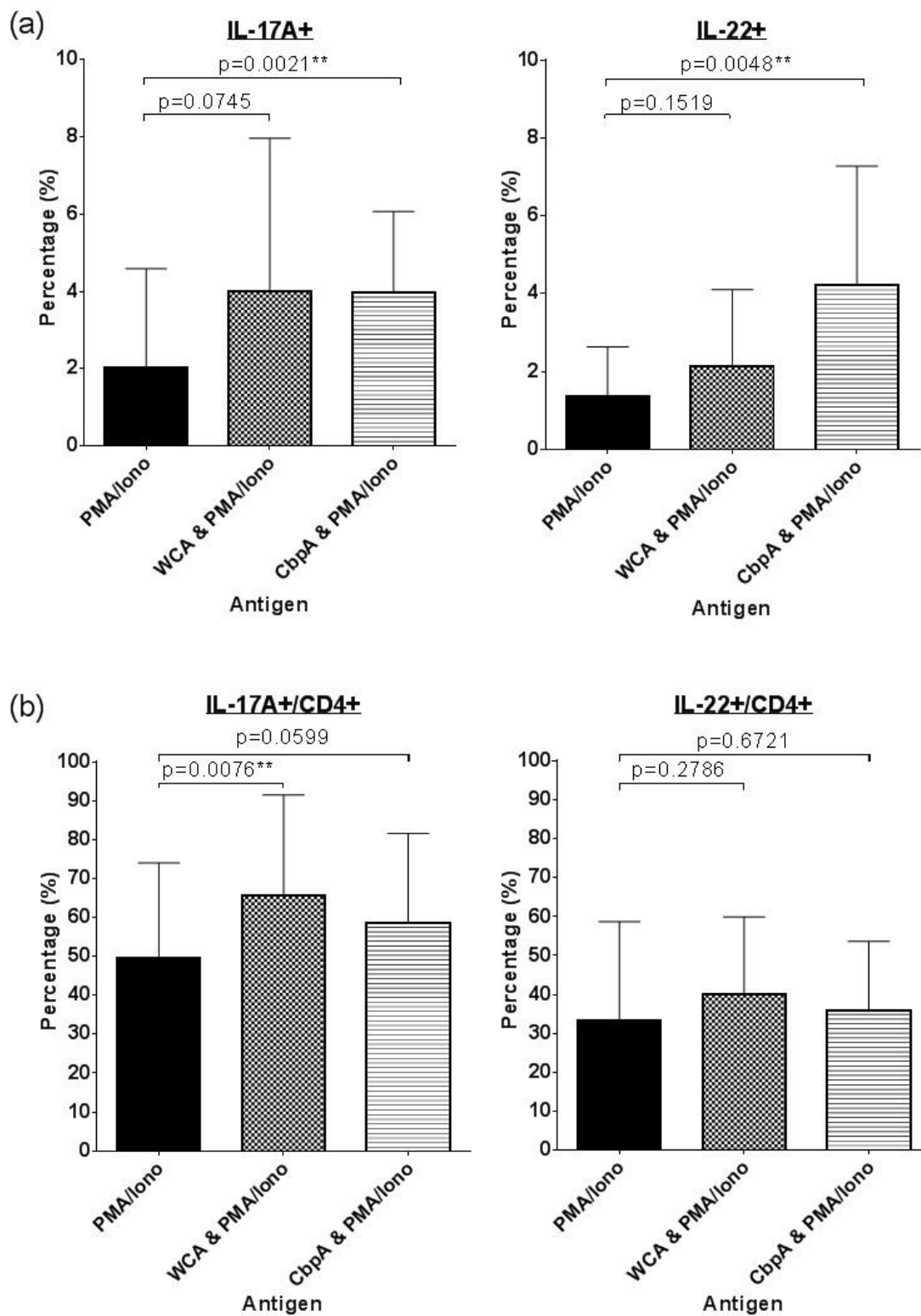
**(b) AMNC**



**Figure 3.** The association between age and IL-17A and IL-22 responses (above background) to WCA in (a) PBMC and (b) AMNC. Each data point represents a child's IL-17A or IL-22 response at day 7 or 11 respectively to WCA, supernatants at both time points were collected from the same well of cells.  $n = 37-39$ . Linear regression lines are shown with corresponding  $r^2$  and  $p$  values.

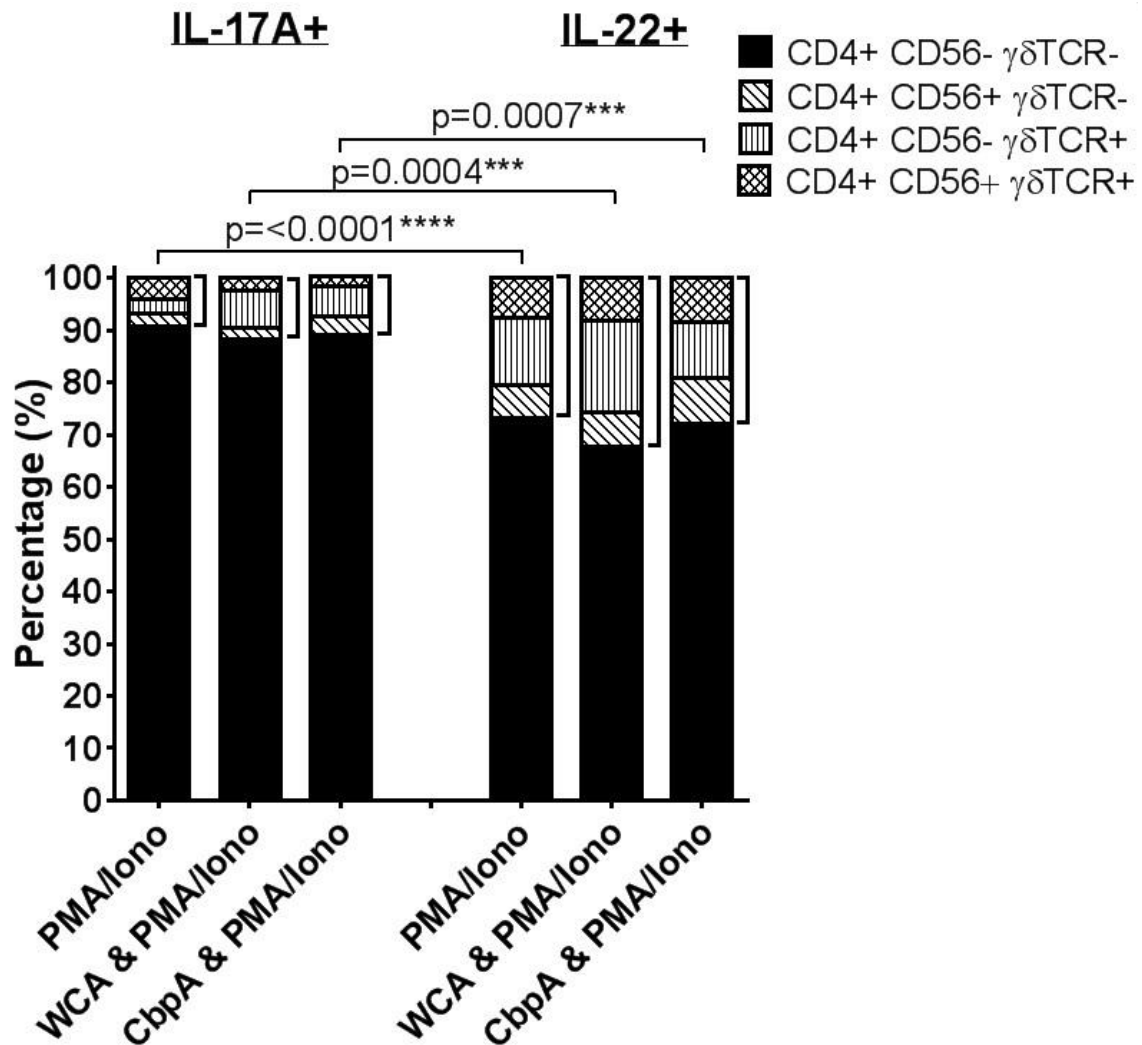


**Figure 4.** Individual day 11 IL-22 responses to WCA (left) and CbpA (right) plotted against corresponding day 7 IL-17 responses in (a) PBMC and (b) AMNC. Values are shown with unstimulated background values subtracted, supernatants at both time points were collected from the same well of cells.  $n=26-38$ . Pearson's correlation coefficients and two-tailed t test p values are shown.



**Figure 5.** Intracellular cytokine staining analysis to identify IL-17A+ and IL-22+ expressing AMNC in response to WCA and CbpA at day 7 and to determine their

CD4<sup>+</sup> expression level. (a) The percentage of live lymphocytes producing IL-17A or IL-22. The average percentage of live cells in the lymphocyte gate was 59.5%, n=14 (data not shown). (b) Comparison of the percentage of cytokine producing live lymphocytes expressing CD4<sup>+</sup> in response to WCA and CbpA. Statistical analysis was conducted using a paired t-test with the p values shown.

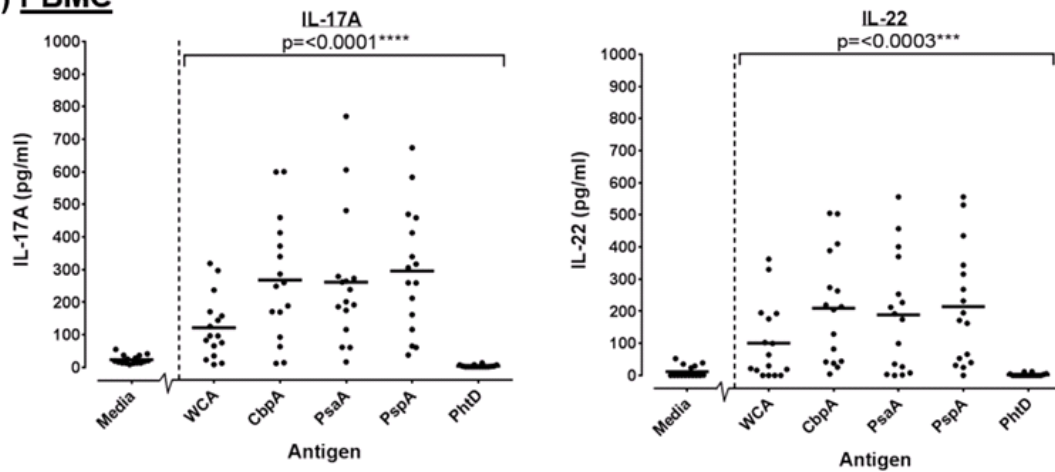


**Figure 6.** Expression of innate cellular markers on CD4+ IL-17A+ and IL-22+ AMNC at day 7. Each bar represents the mean percentage of live CD4+ cytokine producing cells expressing combinations of the cell surface markers CD56 and TCR- $\gamma\delta$  in response to WCA and to CbpA. n=14. Paired t-test was used to compare the IL-17A+ cells expressing combinations of the innate cellular markers, with live IL-22+ cells expressing combinations of the innate cellular markers,. The p values shown relate to the proportions of cells expressing either combination of markers (CD56 or  $\gamma\delta$ TCR or both). Standard deviations for the

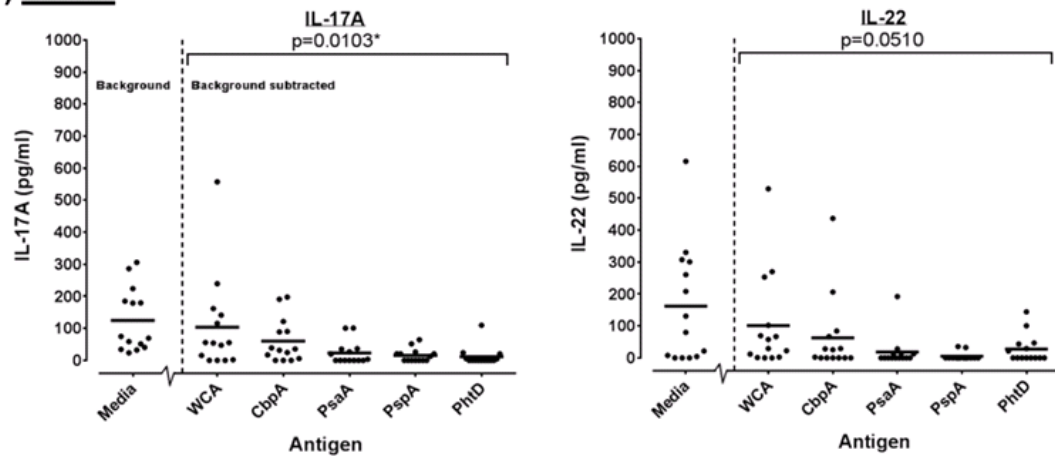
combined innate marker percentages for the six bars are, respectively 15.5; 12.3; 11.9; 21.1; 22.1; 21.3. There are no significant differences between conditions.



**(a) PBMC**



**(b) AMNC**



**Figure 7.** IL-17A and IL-22 responses to WCA and to a panel of pneumococcal antigens – CbpA, PsaA, PspA and PhtD - in (a) PBMC and (b) AMNC. Each data point represents a child's IL-17A or IL-22 response at day 7 or 11 respectively. All PBMC or AMNC samples were tested for all antigens. The background is shown (media) for reference, however the background has been subtracted from the data points showing the responses to each of the stimulations. Supernatants at both time points were collected from the same well of cells. PBMC  $n=16$  and AMNC  $n=14$ . The bar represents the mean. Comparing group means (excluding the media background) was conducted by one-way ANOVA with the  $p$  values shown.

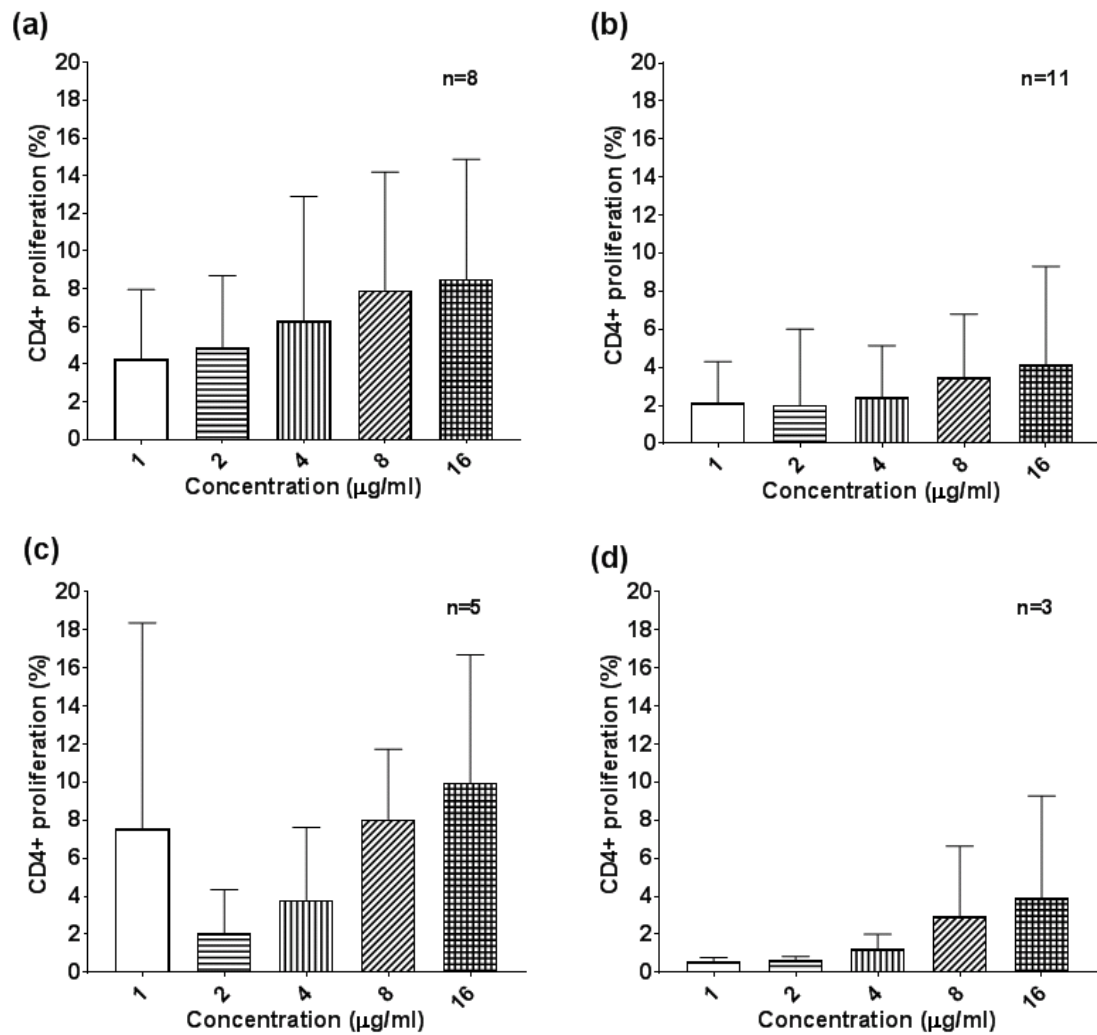
**Supplementary table 1.** Demographic data and history of chronic, recent illnesses and infections in the study population.

Child	Figure(s) data from child is used in	Age (yrs)	Gender (M=Male, F=Female)	Chronic illnesses. Recent illnesses and infections
1	2	3	F	
2	2, 3	1	F	Asthma, Respiratory tract infection
3	2,3	3	M	Otitis media
4	2	6	F	
5	2	4	M	
6	2	5	M	
7	1,3,4	4	F	Asthma
8	3,4	10	F	Urinary retention, Tonsillitis
9	1,3,4	5	M	Tonsillitis
10	1,3,4	5	F	
11	3,4	3	M	Rhinitis, otitis media
12	3,4,7	4	M	Otitis media
13	3,4,7	4	M	Asthma, Tonsillitis
14	3,4,7	2	M	Asthma, Hay fever, Rhinitis
15	3,4,7	6	F	
16	3,4,7	4	M	Asthma
17	3,4	14	F	Tonsillitis
18	3,4,7	5	F	Asthma
19	3,4,7	5	F	Asthma, Otitis media, Tonsillitis
20	3,4,7	4	M	Otitis media
21	3,4,7	4	M	
22	3,4,7	7	F	Upper respiratory infection
23	3,4	2	M	Hypothyroidism, Otitis media
24	3,4,7	4	M	Otitis media, Tonsillitis
25	3,4,7	11	F	
26	3,4	6	M	Asthma, Tonsillitis
27	3,4	8	M	Asperger's Syndrome
28	3,4	11	F	Tonsillitis
29	3,4	2	F	
30	3,4	2	M	
31	3,4	5	F	Juvenile chronic arthritis, uveitis
32	3,4	3	M	
33	3,4	5	M	Tonsillitis
34	3,4	4	M	
35	3,4	3	M	Asthma, Tonsillitis
36	3,4	5	F	CHARGE Syndrome
37	3,4,7	5	M	Otitis media
38	3,4,7	7	M	

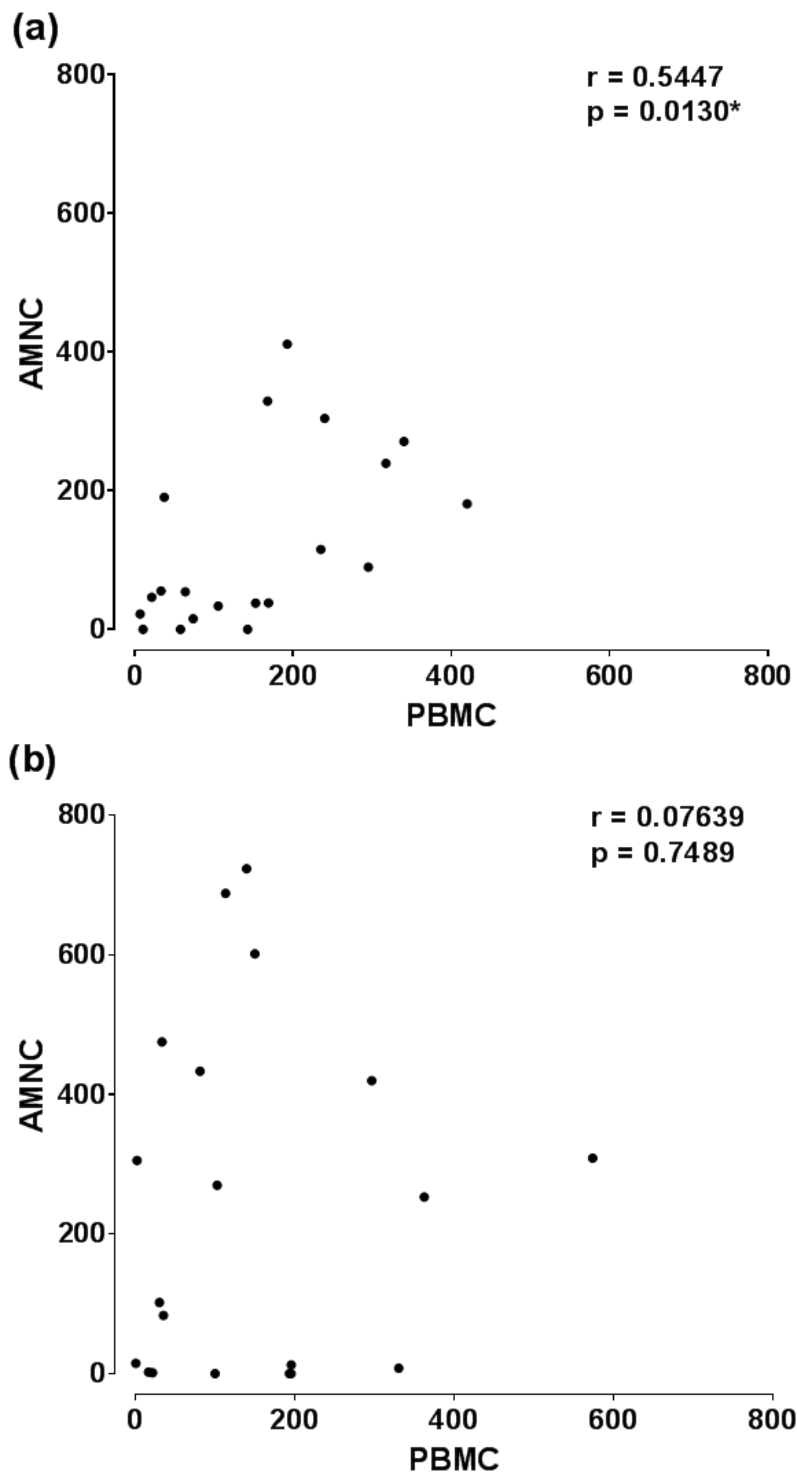
39	3,4,7	3	F	
40	3,4,7	10	F	
41	3,4,7	4	M	Tonsillitis
42	3,4,7	5	M	
43	3,4,7	2	M	Cerebral palsy, chronic lung disease of prematurity, nasal obstruction, Pneumonia
44	3,4,7	2	M	Tonsillitis
45	3,4,7	7	F	Obstructive sleep apnoea
46	3,4,7	3	M	Asthma, tonsillitis, otitis media
47	3,4,7	7	F	Obstructive sleep apnoea
48	3,4	5	F	Obstructive sleep apnoea, varicella
49	3,4,7	2	M	Obstructive sleep apnoea, conductive deafness
50	3,4	3	F	Obstructive sleep apnoea, otitis media
51	3,4	8	M	Obstructive sleep apnoea
52	3,4	3	M	Tonsillitis, otitis media, respiratory tract infections, prematurity, chronic lung disease
53	3,4	3	F	Obstructive sleep apnoea, asthma, tonsillitis
54	3,4	4	F	Obstructive sleep apnoea
55	3,4	10	F	Obstructive sleep apnoea, respiratory tract infections
56	3,4	8	M	Obstructive sleep apnoea, tonsillitis, impetigo
57	3,4	4	F	Tonsillitis, otitis media
58	5,6	1	F	Obstructive sleep apnoea
59	3,4	8	F	Respiratory obstruction, otitis media, deafness
60	3,4	2	F	Obstructive sleep apnoea, otitis media
61	5,6	3	F	Respiratory obstruction
62	5,6	4	M	Obstructive sleep apnoea, previous pneumonia
63	5,6	7	M	Obstructive sleep apnoea, asthma, atopy, sickle trait
64	3,4	2	M	Obstructive sleep apnoea
65	5,6	4	M	Respiratory obstruction, deafness
66	3,4	4	M	Asthma, respiratory obstruction, varicella
67	5,6	3	F	Obstructive sleep apnoea
68	5,6	5	F	Obstructive sleep apnoea, tonsillitis, deafness, otitis media
69	5,6	7	M	Obstructive sleep apnoea, tonsillitis
70	5,6	4	F	Obstructive sleep apnoea
71	5,6	4	M	Obstructive sleep apnoea
72	5,6	5	M	Obstructive sleep apnoea, tonsillitis
73	5,6	5	F	Obstructive sleep apnoea, rhinitis
74	5,6	2	F	Respiratory obstruction, eczema
75	5,6	3	M	Obstructive sleep apnoea, tonsillitis, eczema

### **Footnotes**

- All subjects were reported to be fully immunised apart from child 43 who had had no vaccines.
- Case report form was modified from subject 45 onwards to include indication for surgery - frequently obstructive apnoea.



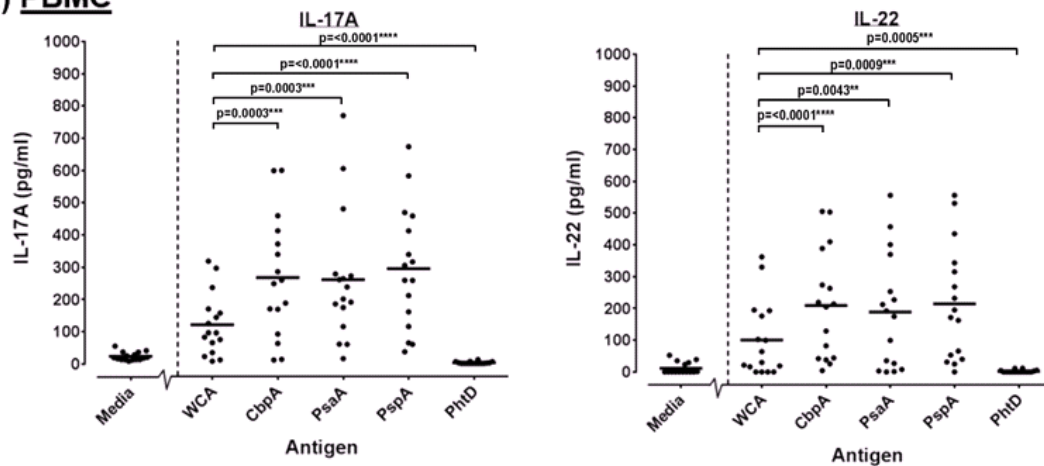
**Supplementary figure 1.** Human nasal associated lymphoid tissue optimal / sub-maximal CD4+ proliferation responses to (a) CbpA, (b) PsaA, (c) PspA and (d) PhtD at day 7. The background unstimulated responses have been subtracted. Bars represent the mean with the standard deviation shown.



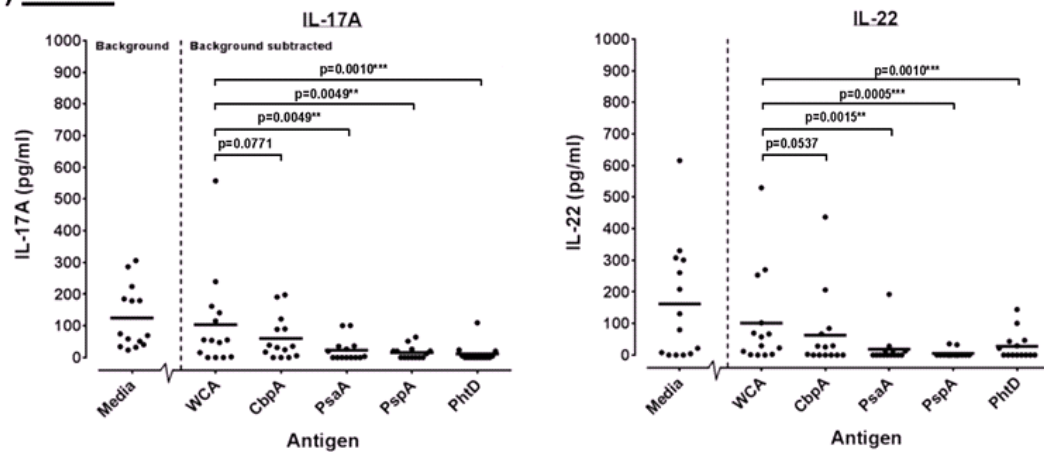
**Supplementary figure 2.** Individual PBMC and AMNC (a) IL-17A and (b) IL-22 responses to WCA. Data points represent a child's IL-17A or IL-22 response at day 7 and 11 respectively. The background unstimulated responses have been

subtracted. Supernatants at both time points were collected from the same well of cells.  $n=20$ . Pearson's correlation coefficients and two-tailed t test p values are shown.

**(a) PBMC**



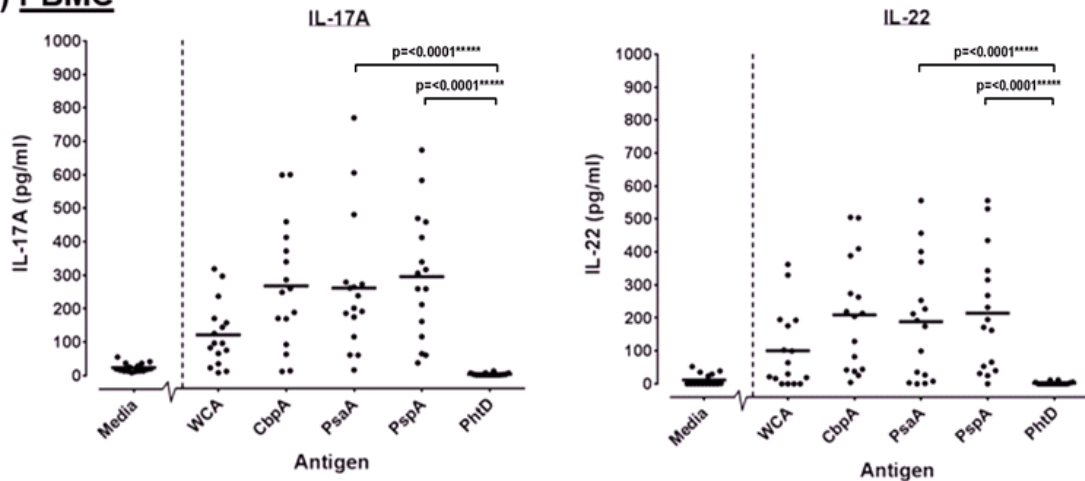
**(b) AMNC**



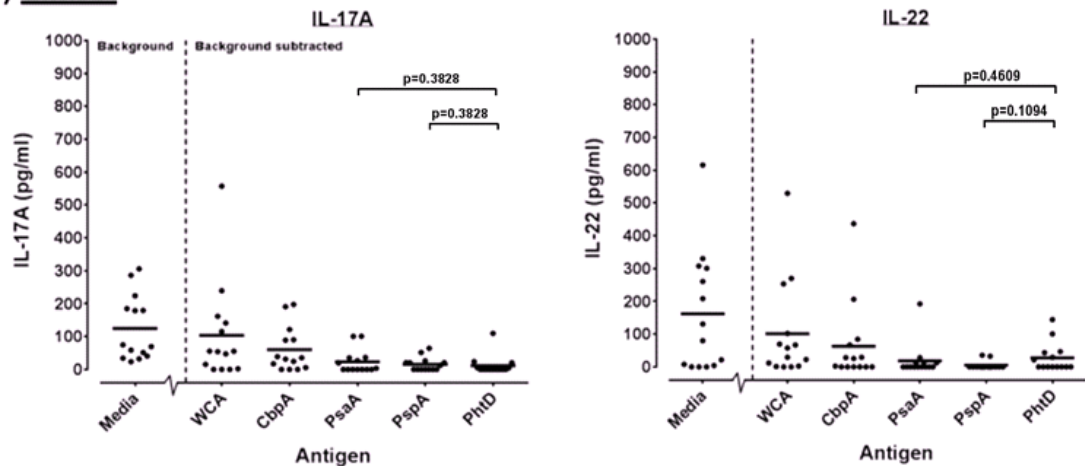
**Supplementary figure 3.** IL-17A and IL-22 responses to WCA and to a panel of pneumococcal antigens – CbpA, PsaA, PspA and PhtD - in (a) PBMC and (b) AMNC. Each data point represents a child's IL-17A or IL-22 response at day 7 or 11 respectively. All PBMC or AMNC samples were tested for all antigens. The background is shown (media) for reference, however the background has been subtracted from the data points showing the responses to each of the stimulations. Supernatants at both time points were collected from the same well of cells. PBMC  $n=16$  and AMNC  $n=14$ . The bar represents the mean. Wilcoxon rank sum test was conducted between WCA and each recombinant protein, p values shown.



**(a) PBMC**



**(b) AMNC**



**Supplementary figure 4.** IL-17A and IL-22 responses to WCA and to a panel of pneumococcal antigens – CbpA, PsaA, PspA and PhtD - in (a) PBMC and (b) AMNC. Each data point represents a child's IL-17A or IL-22 response at day 7 or 11 respectively. All PBMC or AMNC samples were tested for all antigens. The background is shown (media) for reference, however the background has been subtracted from the data points showing the responses to each of the stimulations. Supernatants at both time points were collected from the same well of cells. PBMC  $n=16$  and AMNC  $n=14$ . The bar represents the mean.

Wilcoxon rank sum test was conducted between PhtD and PsaA or PspA, p values shown.